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ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle☆

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ABSTRACT

The bovine rumen undergoes substantial changes in environmental conditions during the animal's feeding cycle, but the effects of these changes on microbial populations have not been examined systematically. Two dairy cows fed a mixed forage/concentrate ration at 12 h intervals over 4 feeding cycles displayed substantial changes in ruminal pH and volatile fatty acid (VFA) concentrations. Automated ribosomal intergenic spacer analysis (ARISA) of solid- and liquid-associated bacterial populations in samples collected at 2, 4, 6, 9, and 12 h after feeding revealed a high degree of bacterial diversity. A total of 155 different amplicon lengths (ALs) were detected across all 83 samples, and 11–74 detected per sample. A substantial proportion (11%) of the ALs was detected in one cow but not in the other. The proportions of ALs that were detected only in the liquid phase or the solid phase were 13.5% and 1.9%, respectively. Correspondence analysis indicated that bacterial community composition differed between cows and between solid or liquid phases, but overall the solid-associated population displayed less change in composition within and across feeding cycles. The data support the notion that cows fed the same diets can have substantial differences in bacterial community composition, and that the solids-associated (biofilm) communities display greater stability than do associated planktonic communities.

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1. Introduction

Studies of microorganisms and their impact on, and interdependence with, various ecological systems have grown dramatically in recent years, owing in large part to advances in molecular characterization techniques. While environments such as freshwaters, sediments, and soils have been the focus of the majority of these studies [1–4], the same techniques can also be employed to investigate complex symbiotic relationships among microorganisms and their mammalian hosts [5–9]. One such environment is the rumen of ruminant animals. Ruminal bacteria perform functions vital to the health and productivity of the host, including the degradation and fermentation of cellulose and other polysaccharides, and the production of volatile fatty acids (VFA), microbial cell protein, and vitamins used by the host as energy sources, protein, and growth factors, respectively [10,11].

Bacterial populations in nature undergo dynamic turnover, although these dynamics are incompletely understood [12,13]. Thus, we would expect that the bacterial community composition (BCC) of the rumen should also display changes in response to changes in environmental conditions. However, the principal forces that influence these bacterial relationships in the ruminal environment and the variations in the diversity of ruminal bacterial populations over time have been largely unexplored. In the rumen, where environmental conditions such as temperature, ionic strength, and redox potential are relatively constant [10,11], the most important driver of bacterial population structure is likely to be the feeding cycle, during which the microbial community is supplied with large but discontinuous input of fermentable energy sources that support microbial catabolism and growth. Several enumeration studies, using culture-dependent techniques, have revealed only modest changes in the total culturable population [14,15] and in the populations of the principal physiological types (glucose-, starch-, pectin-, xylan-, and cellulose-degrading populations [16]) during the feeding cycle. However, changes in the composition of the entire bacterial community (including both cultured and uncultured members) during the feeding cycle have received relatively little attention.

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The purpose of this study was to characterize bacterial population dynamics in the rumen during the diurnal feeding cycle of the dairy cow, through the use of Automated Ribosomal Intergenic Spacer Analysis (ARISA) [1], a community fingerprinting technique that allows profiling of the culturable and currently unculturable residents of a microbial habitat. The ARISA technique was chosen for this study because of its ability to detect a greater extent of bacterial diversity than other techniques [17], and its usefulness in correlating changes in BCC with changes in various environmental factors [2,12]. In addition, its automated nature allows for rapid and efficient analysis of a substantial number of samples, to more accurately observe shifts in community composition over time. Individual peaks generated by ARISA analysis represent amplicons of a specific length, but not necessarily a single sequence (i.e., the peak may represent more than a single phylotype). Thus, ARISA does not provide a quantitative assessment of individual taxa. Nevertheless, comparison of ARISA profiles does permit broad-scale characterization of the extent of differences among different communities, or of changes in individual communities over time [1,12].

2. Materials and methods

Two animals were chosen for this experiment to provide comparison in ruminal environments between individuals. Both were lactating, fistulated Holstein cows housed in indoor tie stalls adjacent to each other. The cows were maintained according to the protocol approved by the University of Wisconsin Animal Care and Use Committee. Cows were provided feed *ad libitum* at 12 h intervals (0500 and 1100) over a 48 h period (i.e., 4 feeding cycles), along with a continuous supply of water *ad libitum*, and were milked at 12 h intervals (0400 and 1600). The ration (32.5% neutral detergent fiber determined after α-amylase treatment, 39.3% non-fiber carbohydrates, and 17.4% crude protein, dry matter basis) was formulated to meet the National Research Council recommendations for lactating dairy cows, and consisted of the following ingredients (dry matter basis): 32.9% corn silage, 32.2% alfalfa haylage, 15.2% dry shell corn, 5.8% whole cottonseed, 4.4% soybean meal, 3.7% dried distiller grains, 2.8% roasted soybeans, 0.5% blood meal, plus supplemental vitamins and minerals. Within each cycle, ruminal samples were collected pre-feed and at 2, 4, 6, 9 and 12 h post-feeding, the last sample corresponding to the pre-feed sample of the next cycle.

Samples were collected medio-ventral in the rumen, using disposable, arm-length polyethylene gloves, and were transferred to pre-warmed thermos bottles that were immediately sealed and brought to the laboratory within 5 min of collection. The collected material was squeezed through 4 layers of cheesecloth into a CO_2-sparged flask. The separated solids and liquids were placed in separate Corning 50 ml centrifuge tubes and stored frozen at −80 °C prior to the analysis of VFA (liquid samples only) and DNA extraction. VFA were determined by HPLC [18]. DNA extraction was performed as described by Weimer et al. [19] using 25 ml of rumen fluid or 25 g of rumen solid for each sample. During the DNA purification procedure, 1 sample (cow 5003, cycle 1, 9 h post-feed liquid sample) was lost, leaving a total of 83 samples for ARISA analysis.

ARISA was performed generally as described by Fisher and Triplett [1], with the following modifications. The oligonucleotide primers used for ARISA were ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCAAC-3′). The primer ends were complementary to the respective positions 1423 and 1443 of the 23S rRNA and positions 38 and 23 of the 16S rRNA of *Escherichia coli* [20]. Primers were synthesized and labeled with Beckman Coulter (Fullerton, CA) WelRed #2 infrared fluorescent dye (Sigma-Proligo) and were reconstituted in water to a working concentration of 400 nM. Template DNA from the ruminal samples was prepared as described previously [19]. DNA concentrations were determined spectrophotometrically and diluted to a working concentration of 10 μg/ml. ARISA PCR reactions were carried out in 20 μl volumes using Promega (Madison, WI) GoTaq Flexi system reagents containing 4.0 μl Mg-free buffer (5 × concentrate), 2.0 μl of 2.5 mM $MgCl_2$, 0.5 μl of 200 μM dNTP mixture, 2.0 μl each of the 2 primers, 2.0 μl of template DNA, 7.0 μl nuclease-free water, and 0.5 μl of Taq polymerase. Molecular biology grade mineral oil (Sigma–Aldrich, St Louis, MO) was added to each tube in amounts sufficient to cover the reaction mixture. PCR reactions were carried out in an Applied Biosystems (Foster City, CA) thermocycler, under the following conditions: 94 °C for 2 min (1 cycle), followed by 30 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 120 s, and finally with 1 cycle of 72 °C for 120 s.

Amplified PCR product was resolved in a Beckman Coulter CEQ8000 capillary electrophoresis Genetic Analysis System by mixing 1.0 μl PCR product with 0.5 μl of Beckman Coulter (Fullerton, CA) WelRed #1 infrared fluorescent dye-labeled DNA standard ladder consisting of 23 sizes of DNA ranging from 50 to 1000 bp (MapMarker 1000, BioVentures, Murfreesboro, TN) and 39 μl sample loading solution (Beckman Coulter). This mixture was then loaded into microtiter plates, molecular biological grade mineral oil was added to cover the liquid surface, and the capillary electrophoresis conducted according to the manufacturer's directions. The run parameters consisted of a capillary temperature of 50 °C, a denaturation temperature of 90 °C for 120 s, an injection voltage of 2.0 kV for 30 s, and a separation voltage of 6.0 kV for 90 min.

The resulting data were imported as an SCF 3.0 file for analysis using GeneMarker (v 1.70) software (SoftGenetics LLC, State College, PA). Settings to detect and quantify the peaks were based on the methods for AFLP analysis specified in the GeneMarker manual. The panel used for comparison was generated automatically using these parameters, and questionable peaks were screened manually. In addition, the software was used to correct "pull-up" between the 2 dyes used, and baseline subtraction and smoothing was performed. All peaks corresponding to amplicon lengths (ALs) of >238 bp were used for analysis. This length cutoff was selected based on our observation that accurate assignment of amplicon sizes using the size standards became difficult with amplicon peaks of <238 bp. Moreover, amplicons of <238 bp would be expected to have ITS sequences of <151 bp, and relatively few such ITS sequences have been identified [21]. The resulting peak area data were tabulated, with individual samples as columns and AL (in bp) as rows. Correspondence analysis was performed using the method of Ludwig and Reynolds [22]. Calculations were performed using custom software written in the C programming language. The first 3 eigenvalues and eigenvectors were found using the power method. Finally, vectors for pH, total mM VFA, and A:P ratio were added by tabulating these values as vectors and ordination points calculated by the method of vector fitting per McCune and Grace [23].

2.1. Statistical analysis

Milk production and composition data from the 2 cows were analyzed using PROC MIXED of the SAS statistical software program, v. 7.0 (SAS Institute, Cary, NC) [24], with cow, milking (AM or PM), and cow × milking interaction modeled as fixed effects, and with milking within cow as repeated measures. Ruminal chemistry (VFA and pH) data were analyzed with PROC MIXED, with feeding cycle, sampling time within cycle, and feeding cycle × sampling time interaction modeled as fixed effects; with cow × cycle and